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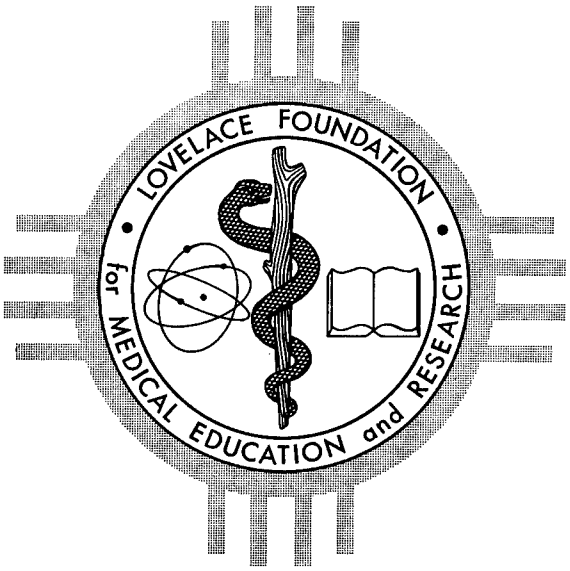

Albuquerque, New Mexico

ULTRAMICRO METHODS IN BIOCHEMISTRY
VII. THE DETERMINATION OF PLASMA OR SERUM UREA NITROGEN
VIII. THE DETERMINATION OF SERUM URIC ACID
IX. THE DETERMINATION OF SERUM CREATININE

by

E. VAN STEWART
AND
BERNARD B. LONGWELL

April 1965

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E. Van Stewart and Bernard B. Longwell

with the technical assistance of Agnes Wood and Jeanette Storrs

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ABSTRACT

Ultramicro methods for the determination of serum urea nitrogen, uric acid, and creatinine have been evaluated and the results are recorded in reports VII, VIII, and IX, respectively.

The method for the determination of urea nitrogen is an ultramicro adaptation of the reaction in which a blue color is developed when the ammonium ion reacts with phenol and hypochlorite. Ammonia is released from urea with urease. The entire reaction is conducted on 5 μl of serum or plasma and the final volume of reaction mixture is 2.205 ml.

The procedure for the determination of uric acid is an ultramicro adaptation of the method which depends on the reduction of phosphotungstic acid in the presence of bicarbonate. The phosphotungstic acid reagent contains lithium sulfate as a stabilizing reagent. The reaction is conducted on 100 μl of protein free solution prepared from 20 μl of serum by precipitation with tungstic acid. Comparative analyses show very good agreement between this method and the macro procedure from which it was derived.

The creatinine method is an adaptation of a standard procedure utilizing the reaction with alkaline picrate. The reaction is carried out on 100 μl of protein-free solution obtained from 40 μl of serum by precipitation of the protein with tungstic acid. The results obtained with the method are in good agreement with values obtained using the macro procedure from which the ultramicro analysis was derived.

Each analytical procedure is evaluated by determination of its reproducibility, and standard deviation analyses are presented.

ACKNOWLEDGMENTS

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VII. THE DETERMINATION OF PLASMA OR SERUM UREA NITROGEN

INTRODUCTION

The determination of blood or plasma urea nitrogen has been generally carried out (a) by the reaction of urea with diacetyl[1] and subsequent measurement of light absorption of the colored complex formed, and (b) by the release of ammonia from urea enzymatically with the specific enzyme, urease[2, 3, 4]. In the latter procedures, the ammonia has been determined titrametrically after trapping it in a standard acid[3] or spectrophotometrically after formation of a colored complex with Nessler's reagent[4]. One of the older methods measured nitrogen gasometrically after its release from urea by alkaline hypobromite[5].

The methods which depend on the reaction of urea with diacetyl are subject to criticism because the final colored complex is photosensitive[6] and, more seriously, the color does not conform to Beer's law[7]. Methods which employ urease have been generally preferred because of the specificity of the reaction. Both of the methods of final measurement of the ammonia have definite, but not insurmountable disadvantages. For example, one method of transferring the ammonia from the reaction mixture to standard acid before titration has utilized aeration of the enzyme mixture after alkalinization. Some investigators have encountered difficulty with quantitative recovery of the ammonia[8] when this procedure was used. Complexing the ammonia directly with Nessler's reagent after the urease reaction has been widely adopted. This approach may be troublesome because of a tendency toward precipitate formation.

Recently, Chaney and Marbach[9] described a procedure which employs the Berthelot reaction of ammonia with phenol and hypochlorite. They utilized the catalytic effect of sodium nitroferricyanide to increase the rate of the reaction and were also able to stabilize the reagents. This procedure employs urease to liberate the ammonia and thus is specific.

The color developed conforms to Beer's law. Altogether the reaction is accurate, reproducible, and technically uncomplicated. This report describes the development of an ultramicro modification of the method of Chaney and Marbach[9] which is reliable and remarkably suited to routine analysis in bulk.

PROCEDURE

A. Reagents

1. Phenol reagent. Dissolve 5 gm of phenol and 0.03 gm of sodium nitroferricyanide in distilled water and make up to 500 ml. Store in the refrigerator. Pour out a small amount for each day's use and replace stock.

2. Hypochlorite reagent. To a solution of 2 gm of sodium hydroxide in distilled water add 10 ml of ($\cong$ 5% available chlorine) sodium hypochlorite. Make up to 500 ml with distilled water and store in the refrigerator. Pour out a small amount for each day's use and replace stock.

3. Urease buffer. Dissolve 10 gm of disodium dihydrogen ethlenediamine tetraacetic acid in approximately 700 ml of distilled water. Adjust the pH to 7.0 using 1.0 N sodium hydroxide. Dilute to 1000 ml with distilled water.

4. Buffered urease reagent. Dissolve 10 mg of urease (Sigma's Type II or Type V) in 10 ml of urease buffer. Prepare fresh daily.

5. Nitrogen standards.

a. Ammonium sulfate (15 mg N/100 ml). Dissolve 70.7 mg dried ammonium sulfate in and make up to 100 ml with distilled water.

b. Urea (15 mg N/100 ml). Dissolve 32.16 mg of dried urea in and make up to 100 ml with distilled water.

B. Procedure

Measurements of the samples, standard, blank, and urease reagents were made with the Sanz type polyethylene pipette[10].

1. To glass test tubes with a capacity greater than 2 ml add 5 μl of blank, standard, or unknown. Use distilled water for the blank.

2. To each tube add 200 μl of buffered urease reagent in such a way that all of the sample is washed to the bottom of the tube with the urease solution. Then incubate at 37°C in a water bath or heating block for 20 minutes (50°C for 4 minutes).

3. Add first to each tube 1.0 ml of phenol reagent and second, 1.0 ml of hypochlorite reagent. Mix thoroughly and let stand at 37°C for 30 minutes to develop color.

4. Transfer the colored solution to 10x75 mm cuvettes and read absorbance at 580 mμ against distilled water in the Spinco spectrocolorimeter that is equipped with a 10x75 mm cuvette adaptor*.

C. Calculation

$$\frac{\text{Absorbance (A) of unknown - A of blank}}{\text{A of standard - A of blank}} \times 15 = \text{mg/100 ml of serum}$$

RESULTS

A. Standard Curve

From Figure 1 it can be seen that a linear relationship is obtained between absorbance and concentration with urea concentrations up to 40 mg/100 ml. The absorbance found at 40 mg/100 ml is at the upper limit where most spectrophotometers can be read accurately. It would, therefore, be more practical to limit the reading range to 35 mg/100 ml.

B. Dilution of Final Color

An important advantage of this method is realized by the fact that it is possible to dilute the final color obtained with water to an acceptable

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*The data presented herein were obtained with the Spinco spectrocolorimeter. The measurement of absorbance has since been transferred to the Beckman DU spectrophotometer modified to become a recording spectrophotometer by attachment of the Gilford (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) recorder, ultramicro adapter, automatic cell positioner and light source control.

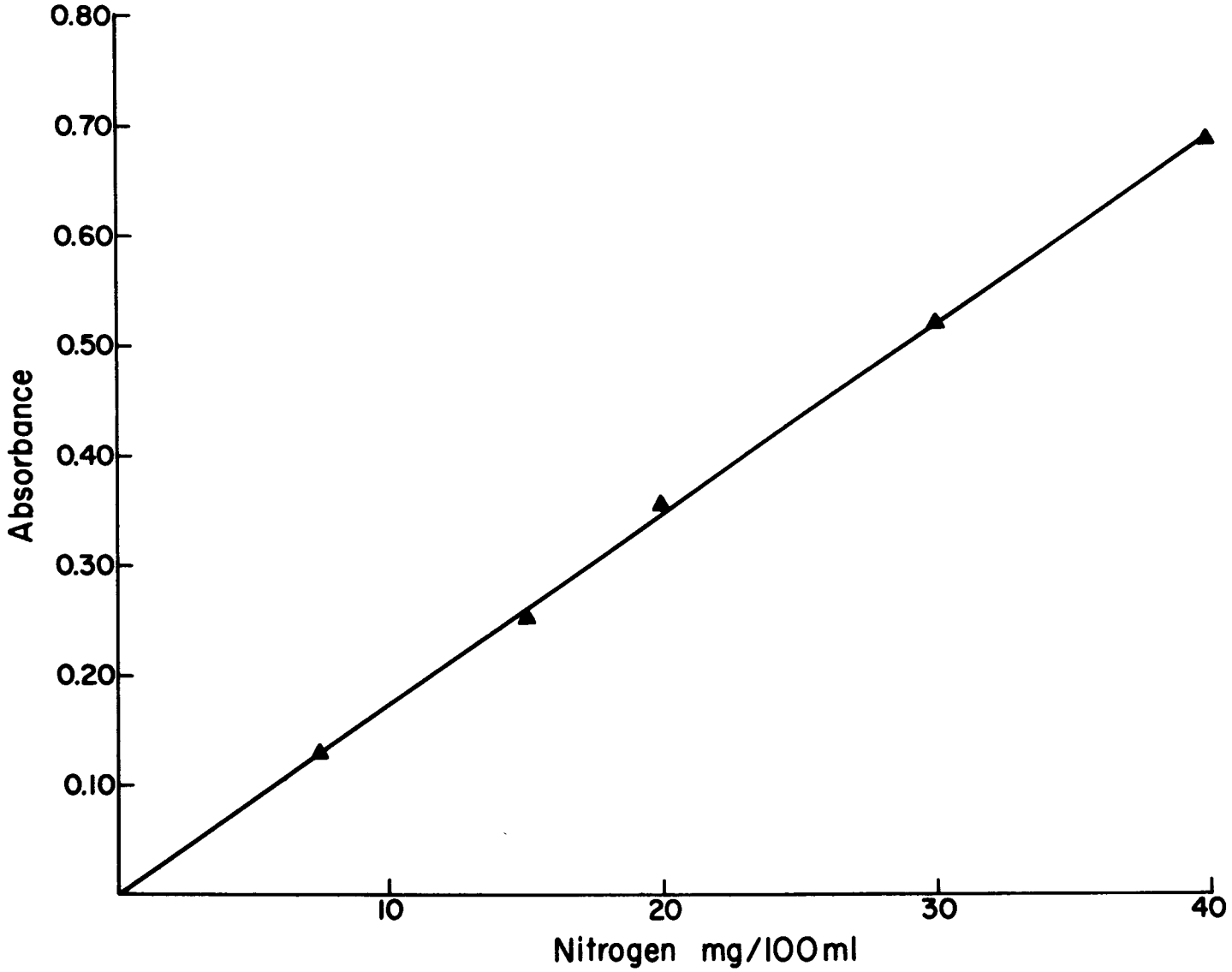

Fig. 1 Standardization Graph.
Ultramicro Determination of Urea Nitrogen.
Ammonium Sulfate Standard.

reading range when the original sample color produced is too intense. Two aliquots of an abnormal control serum were determined by the ultramicro method. One part of the resulting colored solution was diluted with one part of water and mixed thoroughly. One part of the diluted solution was again diluted with one part of water and mixed. The final color absorbance then was determined in Bessey-Lowry micro cuvettes on the Gilford Model 2000 multiple sample absorbance recorder against water. From the results listed in Table 1, it can be seen that dilution of the final color produced can be used with confidence.

C. Comparison with the AutoAnalyzer

The results of this procedure were compared with those of the AutoAnalyzer on 20 serum samples. The AutoAnalyzer determines urea by means of its reaction with 2, 3-butanedione monoxime (diacetyl monoxime). These analyses are listed in Table 2. Good agreement is obtained within the range examined (10.2 to 43.2 mg per 100 ml by the ultramicro method).

D. Standard Deviations and Variance Analyses

Thirty determinations were performed on the same serum sample. A mean of 12.4 $\pm$ 0.23 mg per 100 ml serum with a range of 11.7 to 12.6 mg per 100 ml serum was obtained.

Five serum samples were analyzed in triplicate and the standard deviation calculated by the formula:

$$Sd = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

The samples ranged from 9.4 to 56.4 mg per 100 ml and the calculated standard deviation was $\pm$ 0.67 mg per 100 ml of blood urea nitrogen.

DISCUSSION

A. Mixing of Enzyme and Serum

All glass tubes with a larger than 2 ml capacity that have been used in this laboratory are too long for the sample pipette to reach the bottom

TABLE 1

DILUTION OF FINAL REACTION COLOR

Dilution	Absorbance		mg per 100 ml* BUN	
	Aliquot 1	Aliquot 2	Aliquot 1	Aliquot 2
None	1.160	1.157	52.1	52.0
1:2	0.582	0.579	52.4	52.0
1:4	0.287	0.284	51.6	51.2

Two aliquots of abnormal control serum (Hyland Laboratories, 4501 Colorado Blvd., Los Angeles, California) were determined by the procedure listed. One part of the final colored solution was diluted with one part of distilled water and thoroughly mixed. One part of the diluted mixture was again diluted with one part of distilled water. The original color and dilutions were read at 580 mμ on a Gilford Model 2000 multiple sample absorbance recorder against water.

*The dilution of the blank must be used when calculating the diluted solutions. In this example, one-half of the blank absorbance was subtracted from the absorbance obtained for the 1:2 dilution. The corrected absorbance was then calculated in the described manner and the result multiplied by 2. Similar treatment was given to the 1:4 dilution.

TABLE 2

COMPARISON OF THE ULTRAMICRO METHOD WITH THE AUTOANALYZER DETERMINATION

Sample	Plasma urea nitrogen mg/100 ml	
	Ultramicro	AutoAnalyzer
1	15.5	16.0
2	13.0	14.0
3	12.3	12.0
4	43.2	45.0
5	15.6	16.0
6	13.2	14.0
7	12.1	11.0
8	10.2	9.0
9	14.0	15.0
10	13.2	13.0
11	14.2	13.0
12	16.5	16.0
13	14.2	15.0
14	13.2	13.0
15	25.8	29.0
16	18.3	17.0
17	12.0	10.0
18	15.5	15.0
19	23.8	23.0
20	10.8	10.0

of the tube. This necessitates the deposition of the sample on the sides of the tube. Unless the buffered urease is added in such a way that the enzyme comes in contact and mixes with all of the serum sample, erroneously low results will be obtained. Adequate mixing and washing of the sample with the urease was achieved if the buffered urease reagent was applied to the tube in the same place the sample had been deposited. The volume of buffered urease used appears to be sufficient to rinse the sample satisfactorily from the sides of the tube.

B. Stability of Reagents

The phenol reagent and the hypochlorite reagent have remained active for 60 days when they were stored in the refrigerator and only the amount required for a day's run removed. A fresh urease solution is prepared each day. Chaney and Marbach[1] prepared the urease in a slightly different manner from that described in this method. They state that their urease solution was active for 30 days if kept frozen and only the portion needed removed and thawed.

C. Purity of the Urease

Two grades of urease* have been used with this method. Grade V is a more highly purified preparation than Grade II. Blanks obtained with Grade II urease are from two to three times higher than blanks obtained with Grade V urease. The results obtained when Grade II urease was used appeared to be accurate and reproducible. Although higher blanks were found, no practical disadvantage was encountered with Grade II urease.

D. Wavelength for Reading

The blue color produced by the method described above has a maximum absorptivity at a wavelength of approximately 640 mμ. The recommended wavelength of 580 mμ results in absorbance values which extend the upper

*Sigma Chemical Company, St. Louis, Missouri.

limit of urea concentration which may be measured considerably beyond that possible at 640 mμ. No difficulty has been encountered with this adjustment. However, if quantification at the maximum wavelength is preferred, modification of the procedure by adding more phenol reagent and hypochlorite reagent in equal volumes is quite acceptable. A volume can be found such that the final color developed gives lower absorbance readings (rendered so by dilution) and measurement of absorbance at 640 mμ then becomes possible.

SUMMARY

An ultramicro method for the determination of serum or plasma urea nitrogen has been developed. The method is an adaptation of the procedure of Chaney and Marbach[9] which used the color reaction produced by phenol and hypochlorite after liberation of ammonia from urea with the enzyme urease.

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VIII. THE DETERMINATION OF SERUM URIC ACID

INTRODUCTION

The commonly used methods for uric acid determination have utilized a protein-free filtrate of blood or serum to form a blue color by the reduction of phosphotungstic acid. Carbonate[1], cyanide[2], or silicate[3] have been employed to adjust the reaction mixture to the proper alkalinity. Reduction of phosphotungstate by uric acid is non-specific, and other substances present in the serum will give the reaction[4]. Methods using the enzyme "uricase" and either colorimetric or spectrophotometric determination to detect the quantity of uric acid destroyed per given unit of time have been employed to determine uric acid specifically[5].

It has been estimated by comparison of the specific enzyme methods to the colorimetric methods that from 2 to 15 percent of the color produced by the reduction of phosphotungstic acid is non-specific[6,7]. Although the enzyme methods are more accurate, technically, they are time consuming and are considerably more difficult. It was, therefore, decided that the small amount of error resulting from the non-specific color reaction would be preferable to the additional time required by the enzyme procedures.

Henry, Sobel, and Kim[1] proposed a method using carbonate as the alkalinizing reagent with the addition of lithium sulfate to the phosphotungstate acid reagent to prevent clouding in the reaction mixture. The lithium salts were stated to increase the stability of the reagents, thereby giving more reproducible results. They also, by precipitation of the protein at a more basic pH, were able to eliminate the slight loss of uric acid that sometimes separates with the precipitated protein. When they compared their method to a specific enzymatic method, they concluded that about 11 percent of the color produced was due to non-specific chromogens, but that good correlation existed between the two methods

indicating changes in uric acid concentration. An ultramicro adaptation of the method of Henry, Sobel, and Kim[1] is presented here.

PROCEDURE

A. Reagents

1. Sulfuric acid (0.66 N). Carefully pipet 1.85 ml of reagent grade concentrated sulfuric acid (H_2SO_4) into about 50 ml of water and dilute to 100 ml when cool.

2. Sodium tungstate (10%). Dissolve 10 gm of reagent grade sodium tungstate ($Na_2WO_4 . 2H_2O$) in distilled water and dilute to 100 ml in a volumetric flask.

3. Sodium carbonate (14%). Dissolve 14 gm of reagent grade anhydrous sodium carbonate in distilled water and dilute to 100 ml in a volumetric flask.

4. Phosphotungstic acid. Dissolve 40 gm of reagent grade sodium tungstate ($Na_2WO_4 . 2H_2O$) in 300 ml of distilled water in a flask with a standard size ground glass joint to permit fitting of a glass-joint reflux condenser. Add 32 ml of 85 percent phosphoric acid and reflux gently for two hours. Cool to room temperature. Make up to one liter with water and add 32 gm of lithium sulfate ($Li_2SO_4 . H_2O$) and dissolve. Store in the refrigerator.

5. Stock standard (40 mg/100 ml). Dissolve 24 mg of lithium carbonate, anhydrous, in 40 ml of warm (60°C) distilled water and add to this with stirring 40 mg of uric acid (Matheson, Coleman, and Bell). Transfer quantitatively to a 100 ml volumetric flask using about 30 ml of distilled water for the rinsings. Dilute to 100 ml and store in the dark.

6. Working standard. Dilute one ml of the stock standard to 10 ml with distilled water. Make a fresh working standard for each day's work.

B. Procedure

All measurements were made with the Sanz type polyethylene pipette and the reactions were carried out in 400 μl polyethylene test (centrifuge) tubes[8].

1. Preparation of Filtrates:

	Blank	Standard	Unknown
Water	20 μl		
Standard		20 μl	
Serum			20 μl
Sulfuric acid (0.66 N)	10 μl	10 μl	10 μl
Water	160 μl	160 μl	160 μl
Sodium tungstate (10%)	10 μl	10 μl	10 μl

2. Add in the order stated, then mix thoroughly (Vortex Junior mixer) and centrifuge for one minute.

3. Transfer 100 μl of the supernatant solution to clean tubes and add 40 μl of 14 percent sodium carbonate reagent, followed by 40 μl of phosphotungstic acid reagent.

4. Mix and let stand for 15 minutes (no longer than 30 minutes).

5. Read absorbance at wavelength 680 mμ on the Coleman Junior spectrophotometer, or 650 mμ* on the Spinco colorimeter against water.

C. Calculation

$$\frac{\text{Absorbance (A) of unknown - A of blank}}{\text{(A) of standard - A of blank}} \times 4 = \text{uric acid, mg per 100 ml}$$

RESULTS

A. Standard Curve

In Figure 1, the concentration of uric acid in mg per 100 ml is plotted against the corrected absorbance obtained. The plot is linear to at least

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*The Spinco spectrocolorimeter has a maximum wavelength of 650 mμ. Higher absorbance values are obtained at 680 mμ. It is, therefore, preferable to read at 680 mμ if a Coleman Junior spectrophotometer is available. The data presented herein were obtained with the Coleman Junior spectrophotometer modified for ultramicro analysis by Coleman Instruments, Inc. The measurement of the final color developed has since been transferred to the Beckman DU spectrophotometer modified to become a recording spectrophotometer by attachment of the Gilford (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) recorder, ultramicro adapter, automatic cell positioner, and light source control.

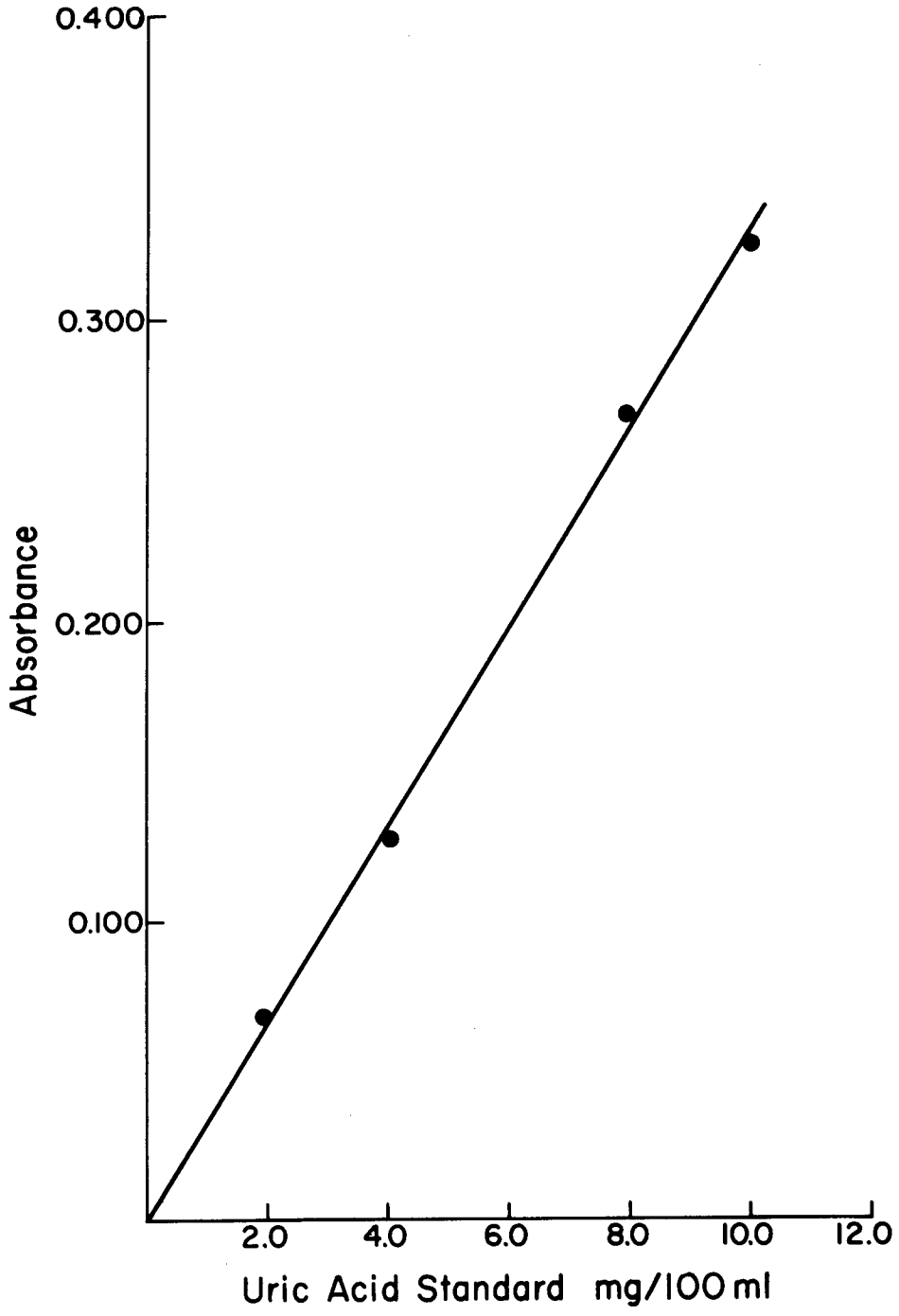

Fig. 1 Standardization Graph.
Ultramicro Determination of Uric Acid.

10 mg per 100 ml which was the highest concentration investigated.

B. Comparison of the Ultramicro Method to the Macro Method

Human serum was analyzed for uric acid by both the macro procedure of Henry et al.[1] and by the ultramicro adaptation of the procedure described here. The results are listed in Table 1. From the values listed, it can be seen that there is good agreement between the two methods.

C. Standard Deviations

Thirty analyses were performed on the same serum sample over approximately one week. A mean of 4.3 mg per 100 ml of serum was found with a standard deviation of ± 0.1 mg per 100 ml of serum.

Five different serum samples ranging from 4 to 11 mg per 100 ml were analyzed in triplicate and the results calculated by the following formula:

$$Sd = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

A standard deviation of ± 0.03 mg per 100 ml of serum was obtained.

DISCUSSION

The ultramicro modification of the uric acid procedure of Henry et al.[1] described herein can be applied with confidence to the determination of uric acid levels at least as high as 10 mg per 100 ml without dilution of the sample. Good reproducibility can be expected as well as good agreement with the macro method. Very few of the serum samples tested produced a noticeable turbidity in the final color reaction and the maximum color produced appeared to be stable for approximately 15 minutes.

The stock standard has, at times, been unstable and decreases in chromogenicity within a day or two after it is prepared as evidenced by decreased absorbance when a fresh dilute standard is carried through the procedure. No explanation can be given for this lability at the present time. For this reason, a fresh stock standard has been prepared weekly in this laboratory whether or not there is evidence of deterioration.

TABLE 1

COMPARISON OF THE MACRO AND ULTRAMICRO METHODS FOR THE DETERMINATION OF SERUM URIC ACID

Sample	Macro	Ultramicro
1	6.5	6.5
2	6.5	6.6
3	6.6	6.7
4	6.3	6.4
5	5.1	5.1
6	6.0	6.1
7	5.3	5.3
8	8.4	8.5
9	7.3	7.3
10	5.8	5.7
11	8.1	8.0
12	8.4	8.4
13	5.8	5.9
14	4.3	4.3
15	4.7	4.8
16	14.6	14.3
17	9.7	9.4
18	7.0	7.1
19	5.6	5.7
20	5.7	5.8

SUMMARY

An ultramicro modification of the procedure of Henry et al.[1] for the determination of uric acid is presented. It requires a 20 μl sample of serum or unknown. A standard curve is presented and the ultramicro method has been compared with the macro method. The precision of the method has been found to be quite satisfactory.

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IX. THE DETERMINATION OF SERUM CREATININE

INTRODUCTION

Most of the methods used to determine creatinine in blood have utilized the Jaffé reaction in which picric acid is reacted with creatinine in an alkaline solution. Although this reaction is widely used, it is generally recognized that substances other than creatinine contribute to the color produced. Among these are ascorbic acid, acetone, and glucose[1], aminohippurate and protein[2]. More of these non-specific chromogens have been found in erythrocytes than in serum or plasma[3]. Therefore, creatinine is determined more accurately if serum or plasma is used in preference to whole blood.

Chromogenic reagents other than those used in the Jaffé reaction have been sought to increase the specificity of the determination. Some of these include 3, 5-dinitrobenzoic acid in an alkaline medium[4], a turbidimetric method using Nessler's reagent[5], potassium 1, 4-naphthoquinone-2-sulfonate[6], and the degradation of creatinine to methylguanidine followed by the Sakaguchi color reaction[7].

Mandel and Jones[4] considered the color reaction with 3, 5-dinitrobenzoic acid to be less practical and no more specific than the Jaffé reaction. The reaction with potassium-1, 4-naphthoquinone-2-sulfonate was found by Cooper and Biggs[8] to give poor reproducibility and high results. A specific method developed by Van Pilsum *et al.*[7] is available if a high degree of accuracy is required. However, Cooper and Biggs[8] consider that this procedure requires too many steps and reagents for a routine clinical laboratory.

Despite the nonspecificity of the Jaffé reaction, methods using it appear to be preferable in the routine clinical laboratory because of their relative simplicity. The error introduced by the interference of non-specific chromogens in this reaction is fairly large at normal levels where it ranges up to 34 percent of the total[4]. However, the error is

reduced to a range of 0.5 to 11 percent of the total at levels above 2 mg per 100 ml[4]. Because of the high percentage of nonspecific color production at low levels, slight changes in creatinine concentration would go undetected, but the interference of these nonspecific chromogens decreases when the creatinine level increases and any significant change due to renal damage should be readily apparent. If a higher degree of accuracy is required, the creatinine may be absorbed on Lloyd's reagent and analyzed by the method of Hare[2]. Although the method is more accurate, it requires more reagents and has more steps and is, therefore, more difficult to perform.

The direct method reported here is an ultramicro adaptation of the method of Bonsnes and Tausshy[9], as applied to blood by Brod and Sirota[10], which employs the color reaction with picric acid.

PROCEDURE

A. Reagents

1. Stock standard. Dissolve 10 mg of creatinine (Matheson, Coleman, and Bell) in 0.1 N hydrochloric acid (HCl). Dilute to 100 ml with 0.1 N HCl.

2. Dilute standard (1 mg/100 ml). Dilute 1.0 ml of stock standard to 50 ml with distilled water. Prepare the dilute standard fresh for each day's work.

3. Sodium tungstate. Dissolve 2.5 gm of reagent grade sodium tungstate ($Na_2WO_4 . H_2O$) in water and make up to 100 ml (2.5%).

4. Sulfuric acid. Add 0.92 ml of reagent grade concentrated sulfuric acid (H_2SO_4) from a graduated pipette to about 50 ml H_2O in a volumetric flask. Dilute to 100 ml with water (0.33 N).

5. Picric acid reagent (0.04 M). Dissolve 9.164 gm of reagent grade picric acid in 900 ml of and dilute to one liter with distilled water.

6. Sodium hydroxide (0.75 N). Dilute 3.8 ml of a carbonate-free saturated solution of sodium hydroxide to 100 ml with distilled water.

B. Procedure

The Sanz polyethylene pipettes and test tubes were used throughout the development of this method[11].

1. To a 400 μl polyethylene test tube add 40 μl of water (blank) or serum (unknown), then add 80 μl of sodium tungstate reagent and 80 μl of sulfuric acid reagent.

2. Mix tubes thoroughly (Vortex Junior mixer) and centrifuge for 30 seconds.

3. Transfer 100 μl of the supernatant solution to clean 400 μl polyethylene test tubes.

4. Prepare the standard by adding 100 μl of the dilute standard to a 400 μl polyethylene test tube.

5. To each tube containing the supernatant solution or the dilute standard add 30 μl of the picric acid reagent and 30 μl of the sodium hydroxide reagents and mix.

6. Allow tubes to stand 20 minutes in the dark.

7. Read absorbance in an ultramicro cuvette against water at a wavelength of 500 mμ with either the Coleman Junior spectrophotometer equipped for ultramicro analysis or the Spinco micro spectrocolorimeter*.

C. Calculation

$$\frac{\text{Absorbance (A) of unknown - A of blank}}{\text{A of standard - A of blank}} \times 1.0 = \text{mg of creatinine per 100 ml of serum}$$

*The data presented herein were obtained with the Coleman Junior microspectrophotometer. The measurement of the final color developed has since been transferred to the Beckman DU spectrophotometer modified to become a recording spectrophotometer by attachment of the Gilford (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) recorder, ultramicro adapter, automatic cell positioner, and light source control.

RESULTS

A. Standard Curve

A plot of absorbance against standard concentration is shown in Figure 1. The plot is linear up to at least 5.0 mg per 100 ml. It is, therefore, permissible to calculate the unknown by use of a standard over this range.

B. Comparison of the Ultramicro Method to the Macro Method

Serum samples from 20 patients were analyzed by both the macro and the ultramicro procedures. The results are listed in Table 1. Good agreement was found between the two methods over the range of values determined.

C. Standard Deviations

Thirty determinations were performed on the same serum sample. The analysis extended over a period of four days. Small aliquots of the serum were frozen when it was originally obtained and these portions were removed from the freezer and thawed when required. A mean of 1.1 mg with a standard deviation of $\pm$ 0.06 mg per 100 ml of serum was found.

Five serum samples ranging from 0.98 to 5.91 mg creatinine per 100 ml of serum were analyzed in triplicate and the standard deviation calculated from the following formula:

$$Sd = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

A standard deviation of $\pm$ 0.03 mg per 100 ml of serum was found.

DISCUSSION

The method was originally developed using a saturated solution of picric acid. This was later changed to a 0.04 M solution because of the greater ease of preparation. No apparent difference was observed when

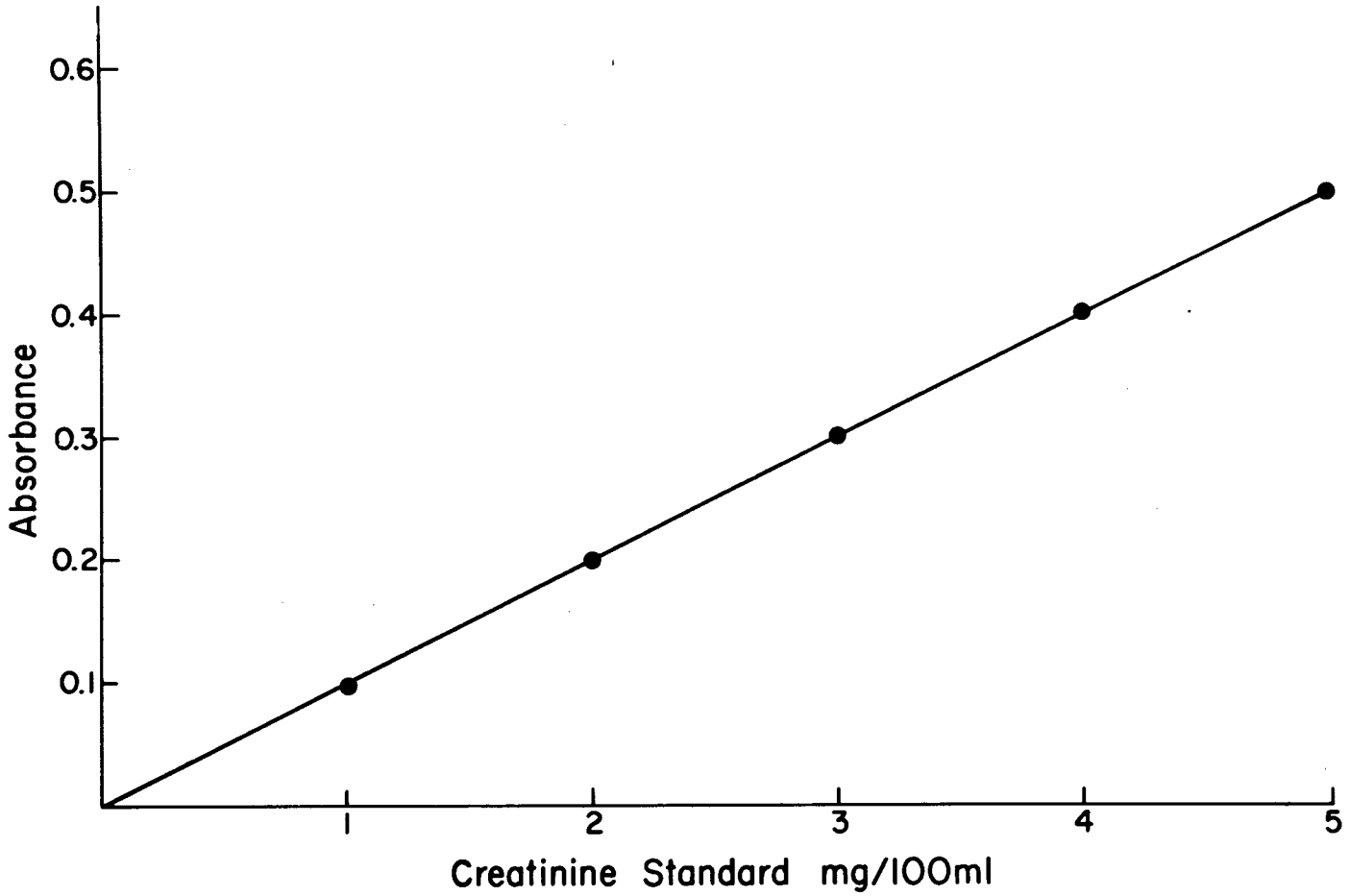

Fig. 1 Standardization Graph.
Ultramicro Determination of Creatinine.

TABLE 1

COMPARISON OF THE ULTRAMICRO METHOD
WITH THE MACRO METHOD

	mg of creatinine per 100 ml of serum	
Sample	Macro	Ultramicro
1	0.70	0.77
2	0.87	0.82
3	13.20	13.30
4	3.93	3.92
5	0.69	0.69
6	1.14	1.19
7	24.10	23.00
8	1.20	1.30
9	1.15	1.14
10	25.20	24.90
11	14.90	14.50
12	7.50	7.55
13	6.00	6.00
14	8.79	8.80
15	5.94	5.93
16	9.42	9.50
17	1.39	1.39
18	4.62	4.56
19	0.75	0.72
20	1.33	1.35

the weaker solution was substituted for the saturated one.

Picric acid appears to stain polyethylene permanently when left in contact with it for even a short period of time. The polyethylene reagent pipette and its storage bottle are soon stained in use. A stained reagent pipette has been used in this laboratory for at least six months without any observed difficulty.

The standard is not carried through the precipitation step in the procedure described. An acid filtrate left after the precipitation of proteins is necessary for the good recovery of creatinine from the serum[9]. When the precipitating reagents are added to the standard solution which is already acid, a filtrate with a higher acidity than that obtained from serum or plasma is produced. This increased acidity in the filtrate from the standard is too high to be adjusted to the pH for optimum color development with the quantity of alkaline picrate used. In order to insure the optimum pH for color development, the standard solution is therefore not mixed with the protein precipitating reagents.

SUMMARY

An ultramicro procedure for the determination of serum creatinine has been developed. The method is an adaptation of the procedure of Bonsnes and Tausshy[9] as modified by Brod and Sirota[10]. The procedure utilizes color developed with picric acid in an alkaline solution. The reaction is non-specific, but its reproducibility and its acceptable correspondence with a similar macro method have been verified.

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